Intein - Blunt protocol

 # PCR to amplify intein old gBlcok

Component	**gBlcok**	**control**
5X PCR Buffer	10 ul	10 ul
dNTPs [10 mM]	1ul	1 ul
Template DNA	1 ul	-
Primer - intein_fw	2.5 ul	2.5 ul
Primer - intein_rev	2.5 ul	2.5 ul
enzyme phusion	0.5 ul	0.5 ul
UPW	32.5 ul	33.5 ul
Total	50 ul	50 ul

Anneling temp: 57 C.

Extension time: 1 min.

 # PNK - Phosphorilation to the old gBlock PCR product

After PCR - the 3' primer is not phosphorylated.

Ingridients:

Component	**Volume**
T4 DNA ligase buffer	2.5 ul
T4 kinase (PNK)	1 ul
DNA	~100 ng
PEG 4000	2.5 ul (5%)
MBW	up to 25 ul
Total	25 ul

- Incubate at 37C for 30 min.
- Heat Inactivation prior to ligation: 65C for 20 min.

- Cool on ice for 10 min.

 # PCR to open Tar1 & 2 for intein1/2

Component	**Tar1-int1**	**Tar1-int2**	**Tar2-int1**	**Tar2-int2**
5X PCR Buffer	10 ul	10 ul	10 ul	10 ul
dNTPs [10 mM]	1 ul	1 ul	1 ul	1 ul
Template DNA				
Primer - fw	2.5	2.5	2.5	2.5
Primer - rev	2.5	2.5	2.5	2.5
enzyme phusion	0.5 ul	0.5 ul	0.5 ul	0.5 ul
UPW				
Total	50 ul	50 ul	50 ul	50 ul

Iclude controls?

Primers:

tar_intein1_fw

tar_intein1_rev

Annealing temp: 67C

tar_intein2_fw

tar_intein2_rev

Annealing temp: 70C

Extension time: 2.15 min

 # Ligation

1. Set up the following reaction in a microcentrifuge tube on ice.
   T4 DNA ligase sould be added last! Ratio of 1:3 vector to insert
   Don't forget to resuspend the buffer at room temp.

   Tip: Since ATP can be damaged by repeated freeze-thaw cycles, it is advisable to make aliquots of the buffer.

Component	Volume (1:3)	Volume (1:5)
10X T4 DNA ligase Buffer	2 ul	2 ul
Vector DNA	50 ng	50 ng
insert DNA - gBlock	60 ng	100 ng
Nuclease-free water (or MBW??)	up to 20 ul	up to 20 ul
T4 DNA ligase	1 ul	1 ul
Total	20 ul	20 ul

The DNA ampunt were calculated in NEB calculator:

http://nebiocalculator.neb.com/#!/ligation

Insert length: 1575 bp

Vector length: 3937 bp

Vector DNA mass: 50 ng

protocol from: https://www.neb.com/protocols/1/01/01/dna-ligation-with-t4-dna-ligase-m0202

2. Gently mix the reaction by pipettting up and down.

3. For blunt ends: incubate at 4C O/N (Low temp is recomended to make it easier for the insert and pasmid to collide and ligate more efficient. O/N cause in this temp the enzyme work slowly).

5. Chill on ice and transform 1-5 ul of the reaction into 50 ul component cells.

 # Screening method - Colony PCR:

Component	Volume	Control
PCR mix	5 ul	5 ul
Colony	1 colony	-
Primer fw	1 ul	1 ul
Primer rev	1 ul	1 ul
UPW	3 ul	3 ul
Total	10 ul	10 ul

Primers:

Mid_tar_fw

Mid_Tar_rev

Expected size: intein1 : 935 bp. intein2 : 986 bp

Annealing temp: 59C

Extension time: 1.15 min

3 posibilities:

1. Self ligation: No bands.
2. Right orientation - 2 bands: 643 bp, 3294 bp
3. Wrong orientation - 2 bands: 643 bp, 3294 bp

The colonies that shows 2 bands (hopefully) will be verified with restriction enzymes to verify the orientation.

Restriction verification:

With KpnI-HF enzyme

CutSmart Buffer

Component	**Volume**	**control - original plasmid Tar1/2**
Buffer X10	2 ul	2 ul
DNA - 1 microgram		
KpnI-HF	1 ul	1 ul
MBW	up to 20	up to 20 ul
Total	20 ul	20 ul

The enzyme appears in 2 places in the plasmid: in the intein and outside the intein.
Expected size:

Tar1-int1: 2 bands: 2040, 3474.

Tar1-int2: 2 bands: 2091,3423.

Tar2-int1: 2 bands: 2040,6472.

Tar2-int2: 2 bands: 2091,3421.

Tar2 – control: 1 band: 5512